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Identification of macrophage migration inhibitory factor in murine neonatal calvariae and osteoblasts

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SUMMARY

Bone resorption and formation are dynamic processes that occur in both normal and injured bone tissues. Regulation of these processes is mediated at the local level by cytokines and growth factors. Macrophage migration inhibitory factor (MIF) is one of the proinflammatory cytokines that activates macrophages and regulates production of other cytokines, such as tumour necrosis factor-α and interleukin-1. We here demonstrate, by reverse transcription–polymerase chain reaction, high expression of MIF mRNA in murine osteoblasts obtained from mouse neonatal calvariae and murine osteoblastic MC3T3-E1 cells. The presence of MIF protein in the osteoblasts was confirmed by Western blot analysis using anti-rat MIF antibody. Moreover, the immunohistochemical study revealed that MIF was localized largely in the cytoplasm. The pathophysiological function of MIF remains undefined; however, the present results suggest that MIF takes part in the osseous metabolism as well as in immunological events.

INTRODUCTION

Bone tissue repeats resorption and formation for its remodelling, which is regulated by numerous cytokines, growth factors and hormones. Bone remodelling exhibits a specific phase in which mononuclear cells are recruited. In this phase, macrophages produce the various bioactive substances regulating the bone resorption and formation.[1–9] In most cases, cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), stimulate bone resorption, whereas growth factors, e.g. transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF), stimulate bone formation. In the bone metabolism, macrophages are considered to play an important role. Infiltration of macrophages are observed within the callus in fracture repair throughout the whole process of bone remodelling.[10,11] This finding strongly suggests that the recruited macrophages are deeply involved in the bone resorption and formation; however, the precise pathophysiological functions of mononuclear cells in the normal or pathological state of bones remain undefined.

The recruitment of macrophages from the peripheral vasculature is induced by chemoattractants released by infiltrating mononuclear cells. In the process of recruitment, macrophage migration inhibitory factor (MIF) was the first lymphokine reported to prevent the random migration of macrophages out of capillary tubes.[12] The protein was considered to be expressed exclusively in activated T lymphocytes; however, a recent report indicated that macrophages are another source of the protein.[13] Human MIF cDNA was the first one cloned, and it was found that MIF consists of 114 amino acid residues.[14] Recently, we cloned rat MIF cDNA, and reported its physiochemical properties.[15–17] Moreover, we succeeded in the crystallization of both human and rat MIF, and revealed the tertiary structure of rat MIF at 2·2 Å resolution.[18–20]

We here report for the first time, by means of reverse transcription–polymerase chain reaction (RT–PCR), Western blot analysis and immunohistochemistry, that MIF is expressed and present in murine osteoblasts. The precise pathophysiological function of MIF remains undetermined; however, the present results suggest the possibility that MIF produced by osteoblasts is profoundly involved in regulation of the bone metabolism.

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MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources. Nitrocellulose membrane filters were purchased from Millipore (Bedford, MA); Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); M-MLV reverse transcriptase and dispase from Gibco (Grand Island, NY); Taq DNA polymerase from Perkin-Elmer (Norwalk, CO); Tissue-Tek OCT compound from Miles Scientific (Naperville, IL); horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody and Micro BCA protein assay kit from Pierce (Rockford, IL); 4-chloro-1-naphthol substrate for HRP from Promega (Madison, WI); Konica immunostaining HRP-1000 from Konica (Tokyo, Japan); complete Freund's adjuvant (CFA), incomplete Freund's

adjuvant (IFA), 3,3′-diaminobenzidine tetrahydrochloride from Wako (Osaka, Japan); alkylacrylates from Merck (Darmstadt, Germany); Histofine SAB-PO kit from Nichirei (Tokyo, Japan), and Protein A Sepharose from Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade.

Antisera

Polyclonal anti-rat MIF serum was generated by immunizing New Zealand White rabbits by recombinant rat MIF as described.[20,21] Recombinant rat MIF was expressed in *Escherichia coli* and purified to homogeneity as previously described.[16] The immunoglobulin G (IgG) fraction (4 mg/ml) was prepared using Protein A Sepharose according to the manufacturer's protocol. Since only a single amino acid residue is different between rat and mouse MIFs,[15] the anti-rat MIF immunologically cross-reacted with mouse MIF.

Osteoblasts

Murine primary osteoblasts were obtained from 1 to 2-day-old ICR murine calvariae.[22] In brief, 30–40 calvariae were stirred for 20 min in 10 ml of α-modified Eagle's medium (α-MEM) containing 0·1% collagenase and 20 000 units of dispase. The dispersed cells were collected by centrifugation at 100 ***g*** for 10 min. The extraction was repeated five times, and these cells were combined and washed with α-MEM. The collected cells were further cultured in α-MEM containing 10% fetal calf serum, and the medium was changed every 3 days. The cells were harvested at days 6 and 12 by phosphate-buffered saline (PBS) containing 0·2% trypsin and 0·02% ethylene diamine tetra-acetic acid (EDTA), and stored at −80° until use. The purity of osteoblasts was found to be more than 95% by examination using alkaline phosphatase staining.

Cell culture

Murine osteoblastic MC3T3-E1 cells were generously provided by Dr H. Dohi of Ohu University Dental School (Fukushima, Japan). The cells were cultured at 37° in a CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% fetal bovine serum (FBS), and subcultured every 3 days. The cells (3×10^4 cells) were cultured for the designated times in 35-mm plastic dishes containing 2 ml α-MEM with 10% FBS. For experiments, cultured cells were rinsed with PBS and the medium was exchanged for medium containing 0·1% bovine serum albumin (BSA).

RT–PCR

The total RNAs were extracted from the primary calvaria osteoblasts and MC3T3-E1 cells with an Isogen RNA extraction kit. The reverse transcription of the total RNA was carried out by M-MLV reverse transcriptase using oligo-dT primers followed by amplification using Taq DNA polymerase. PCR was carried out for 25 cycles of denaturation at 94° for 1 min, annealing at 53° for 2 min and extension at 72° for 1 min using a thermal cycler (Perkin-Elmer, Model 2400). Murine MIF primers used were 5′-TCCGTGCCAGAGGGGTTTCTC-3′$^{40-60}$ (forward) and 5′-AGAACCGCAACTACAGTAAGC-3′$^{215-235}$ (reverse). β-Actin primers used were 5′-ATGGATGACGATATCGCTG-3′$^{1-20}$ (forward) and 5′-ATGAGGTAGTCTGTCAGGT-3′$^{551-570}$ (reverse). After PCR, the amplified products were analysed by agarose gel electrophoresis.

Western blot analysis

The osteoblasts obtained from murine calvariae and MC3T3-E1 osteoblastic cells were disrupted with a Polytrone homogenizer (Kinematica), and the homogenates were dissolved in 20 μl of Tris–HCl 50 mM, pH 6·8 containing 2-mercaptoethanol (1%), sodium dodecyl sulfate (SDS) (2%), glycerol (20%) and bromophenol blue (BPB) (0·04%), and heated at 100° for 5 min. The samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described.[23] The proteins on the gel were electrophoretically transferred onto a nitrocellulose membrane at 50 mA for 1 hr using a semi-dry blot transfer apparatus (Bio-Rad). The nitrocellulose membrane was intensively washed with PBS, and incubated with anti-rat MIF polyclonal antibody (1:1000 in dilution) at room temperature for 1 hr, and further reacted with peroxidase-conjugated anti-rabbit IgG serum (1:1000 in dilution) at room temperature for 1 hr. After the reaction, proteins were visualized with a Konica immunostaining MRP-1000 kit as recommended in the manufacturer's protocol. Protein concentration was determined with a Micro BCA protein assay reagent kit.

Immunohistochemistry

Murine calvariae were embedded and frozen in Tissue-Tek OCT compound, and kept at −80° until use. Six-micrometre thick cryostat sections were fixed in acetone and treated with 10% normal goat serum for 20 min. After washing three times with PBS for 5 min, the tissues were further incubated with 0·3% H_2O_2 for 20 min at room temperature to block the endogenous peroxidase. On the other hand, osteoblasts from the murine calvariae and MC3T3-E1 cells were placed on a glass slide, fixed in 4% paraformaldehyde in PBS at 4° for 10 min, and dehydrated in ethanol for 5 min. The cell samples were immersed in 0·3% H_2O_2 in methanol for 30 min to quench the endogenous peroxidase. For detecting MIF, the calvaria and osteoblast samples were incubated overnight at 4° with anti-rat MIF polyclonal antibody. The samples were stained with an avidin–biotin–peroxidase complex procedure using a Histofine SAB-PO kit according to the manufacturer's protocol. The samples were also counterstained with Meyer's haematoxylin. Non-specific staining was blocked by incubation with normal goat serum (10%) for 10 min.

Enzyme-linked immunosorbent assay (ELISA)

The MIF antibody dissolved in 50 μl of PBS was added to each well of a 96-well microtitre plate, which was then left for 1 hr at room temperature. The plate was washed three times with PBS. All wells were filled with PBS containing 1% BSA for blocking and left for 1 hr at room temperature. After removal of the blocking solution, samples were added in duplicate to individual wells and incubated for 1 hr at room temperature. Next the plate was washed three times with PBS containing Tween 20 (0·05%) (washing buffer) and 50 μl of biotin-conjugated MIF antibody. Following incubation for 1 hr at room temperature, the plate was again washed three times with the washing buffer. Then avidin-conjugated goat anti-rabbit IgG antibody was added to individual wells and incubated for 1 hr at room temperature. After the plate was washed three times, 50 μl of a substrate solution containing 200 μg of *o*-phenylethylenediamine and 10 μl of 30% hydrogen peroxide in 10 μl of citrate–phosphate buffer (pH 5·0) was added to each well. After incubation for 20 min at room temperature, the reaction was

stopped with 50 μl of 1 N sulfuric acid. The absorbance at 492 nm was measured using an ELISA plate reader (Bio-Rad, Model 3550).

RESULTS

Expression of MIF mRNA

MIF mRNA expression in the primary murine neonatal calvaria osteoblasts and MC3T3-E1 cells was examined by RT–PCR analysis. Two micrograms of the total RNA was subjected to reverse transcription. From the cDNA library, the specific transcript for mouse MIF was amplified by PCR. At the expected molecular size (196 bp), MIF mRNA products were detected on the agarose gel (Fig. 1). The relative intensities of the bands normalized by β-actin PCR products did not show any appreciable difference between the primary osteoblasts and MC3T3-E1 cells. No detectable amount of amplified transcript of MIF was observed on murine polymorphonuclear cells (PMN) in the RT–PCR analysis (data not shown). This result indicated that MIF mRNA was constitutively expressed in the osteoblasts.

Western blot analysis

To further confirm the expression of MIF protein by the primary calvaria osteoblasts and MC3T3-E1 cells, Western blot analysis was carried out. The transferred protein bands visualized by a Konica immunostaining HRP-1000 kit migrated to the corresponding molecular mass of MIF, about 12·5 kDa (Fig. 2). In contrast, cell lysates obtained from purified PMN exhibited no detectable amount of MIF protein (data not shown) as previously reported.[13] This result indicated that MIF was produced by both the primary osteoblasts and MC3T3-E1 cells as demonstrated by mRNA expression, and that MIF was synthesized *de novo* in the murine osteoblasts.

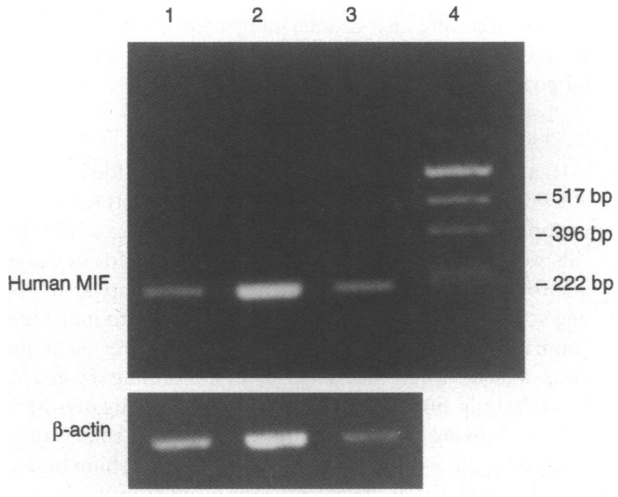

Figure 1. RT–PCR analysis of MIF mRNA of murine osteoblasts. RT–PCR was carried out as described in Materials and Methods. The PCR products were electrophoresed on 2% agarose gel. Lane 1, murine T lymphocytes; lane 2, osteoblasts (day 6); lane 3, MC3T3-E1 cells; lane 4, molecular size marker (pUC18 DNA/Hinf1). The RT–PCR product of β-actin is shown at the bottom of each lane.

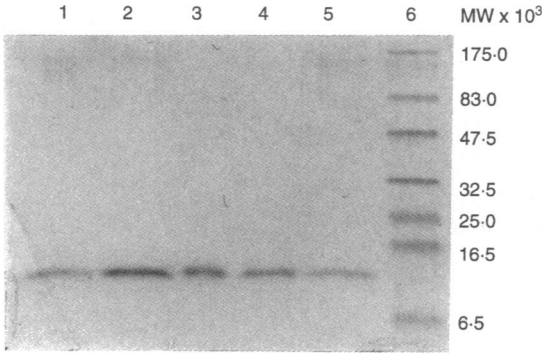

Figure 2. Western blot analysis of murine osteoblasts for MIF. The osteoblasts (5×10^5 cells) were collected, electrophoresed, transferred to a nitrocellulose membrane, and visualized by a Konica immunostaining HRP-1000 kit as described in Materials and Methods. Lane 1, mouse T lymphocytes (3×10^6 cells); lane 2, the primary calvaria osteoblasts (day 6); lane 3, the primary calvaria osteoblasts (day 12); lane 4, MC3T3-E1 cells (5×10^5 cells); lane 5, recombinant rat MIF (50 μg); lane 6, the molecular size marker (Biolabs).

Immunohistochemistry

Immunohistochemical analysis of the neonatal murine calvaria clearly showed the presence of MIF in osteoblasts (Fig. 3a). This appeared as mononuclear cell sheets on the outer surface of the cartilage tissue as described.[24] The cell type was confirmed by alkaline phosphatase staining of serial sections, which showed a strong positive reaction for these cells (Fig. 3b). No positive staining was observed in the calvaria in the reaction with pre-immune rabbit IgG (Fig. 3c) or with the addition of an excess amount of MIF in this staining procedure (data not shown). Furthermore, we carried out immunohistochemical study for the primary osteoblasts obtained from the calvariae and MC3T3-E1 cells. Positive MIF staining was observed within the cytoplasm of osteoblasts (Figs 4a and 5a). This staining procedure did not result in any specific staining in the reaction with pre-immune rabbit IgG (Figs 4b and 5b) or with the addition of an excess amount of MIF in this staining procedure (data not shown). Considering these results together, we concluded that MIF produced by murine osteoblasts was localized in the cytoplasm.

Contents of MIF in the culture medium

MIF concentrations in the culture media of the primary culture osteoblasts and MC3T3-E1 cells at 24 hr were measured by ELISA. It showed that a substantial amount of MIF existed in the media of the primary culture osteoblasts and MC3T3-E1 cells, 6·8 ± 0·8 and 3·5 ± 0·5 ng/ml in 5×10^4 cells/ml, respectively. This indicated that MIF was not only present in the cytoplasm, but also that part of the MIF was secreted into the extracellular space.

DISCUSSION

In this study, we demonstrated for the first time that MIF mRNA was expressed in murine osteoblasts by RT–PCR. The presence of MIF protein in the osteoblasts was further confirmed by Western blot analysis and immunohistochemistry. To date,

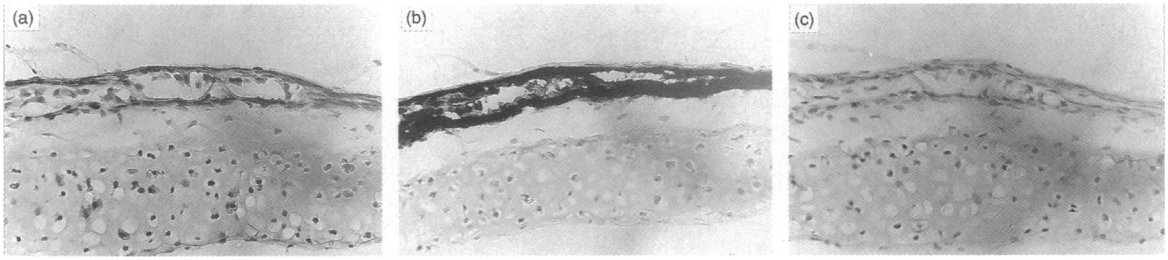

Figure 3. Immunohistochemistry of the murine calvariae by anti-rat MIF antibody. The calvariae were stained with a Histofine SAB-PO kit as described in Materials and Methods. (a) calvariae obtained at 2 days after birth (× 80); (b) alkaline phosphatase staining (× 80); (c) the tissue specimen reacted with pre- immune rabbit IgG as a control (× 80).

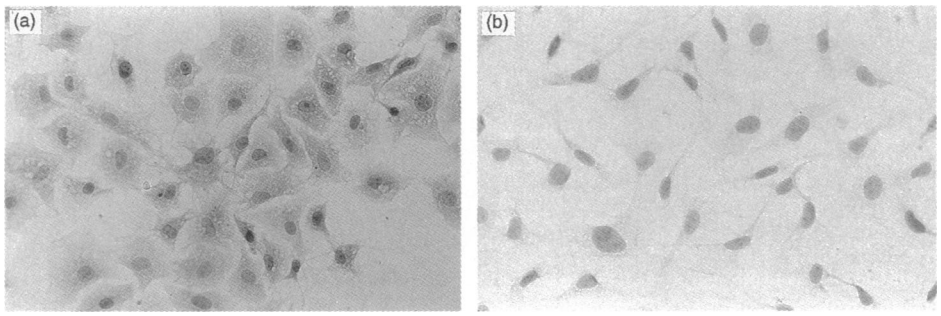

Figure 4. Immunohistochemistry of the murine osteoblasts by anti-rat MIF antibody. The osteoblasts obtained from the murine calvariae were stained with a Histofine SAB-PO kit. (a) the cells obtained at 2 days after birth (× 100); (b) the tissue specimen reacted with pre-immune rabbit IgG as a control (× 100).

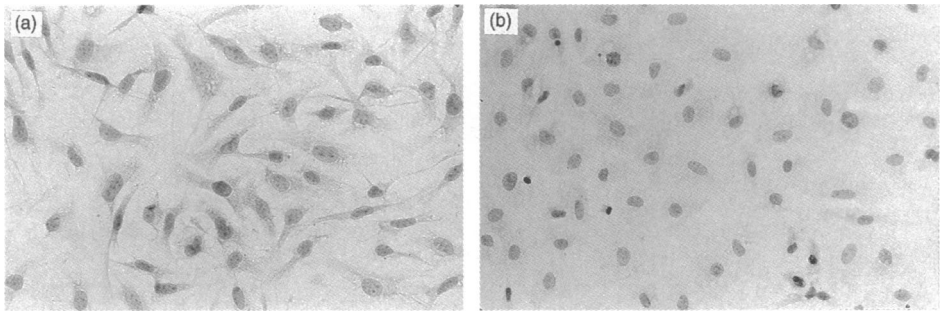

Figure 5. Immunohistochemistry of MC3T3-E1 osteoblastic cells by anti-rat MIF antibody. (a) the osteoblastic cells reacted with anti-rat MIF polyclonal antibody and stained with a Histofine SAB-PO kit (× 100); (b) the osteoblastic cells reacted with pre-immune rabbit IgG as a control (× 100).

it has been reported that MIF stimulates macrophages to secrete other proinflammatory cytokines, e.g. IL-1β, TNF-α,[25,26] and that these cytokines are profoundly involved in the bone metabolism.[27] This fact indicates that MIF secreted by osteoblasts may regulate the bone resorption and formation as a novel member of the regulatory cytokines. However, the precise pathophysiological function of MIF in the bone metabolism still remains unclear.

To date, MIF is known to be present in highly proliferative tissues, such as the early embryonal chicken lens[28] and the basal cell layers of human skin epidermis and corneal epithelium[21,29] From these findings, it is considered that MIF might be profoundly associated with cell proliferation and differentiation. That is, MIF may have a function to stimulate cell proliferation and proliferation required for bone remodelling. The major growth factors synthesized by the osteoblasts include insulin-like growth factor, TGF-β, and PDGF,[30–32] which induce the subsequent stage of the remodelling cycle.[33] Since we found that MIF mRNA was up-regulated by PDGF (unpublished observation), it is very likely that MIF may stimulate cellular proliferation in concert with growth factors.

In contrast, osteoclasts resorb minerals and matrices during the reversal phase of bone resorption in the sequence of bone remodelling. It is of interest that macrophages have the potential to differentiate into osteoclasts when the cells are cocultured with stromal cells.[9] Thus, accumulation of macrophages and osteoblasts around the diseased regions is considered to be important in the pathophysiology of chronic destructive bone diseases, e.g. osteoporosis and rheumatoid arthritis. Taken together, the present results suggest that MIF

secreted by osteoblasts may also take part in the pathogenesis of these destructive diseases through direct or indirect osteoclast stimulation.

At the sites of bone injury and bone remodelling, significant mononuclear cell recruitment occurs. For example, macrophages and osteoblasts are abundant at early stages of fracture healing, and continue to be present through the later stages of healing. These cells secrete a variety of cytokines and growth factors which are essential for bone repair during the initial stage of healing.[34] Despite the importance of macrophages and osteoblasts in the osseous metabolism, little is known about the regulatory mechanism of macrophage recruitment. From the data available to date, it appears that monocyte chemoattractant protein-1 (MCP-1) may contribute, in part, to the recruitment.[35] The interaction between MIF and MCP-1 has not been described; however, identification of MIF in osteoblasts may illustrate a new aspect of the mechanism of macrophage recruitment in fractured bone tissues.

Recently, MIF was found to act to override glucocorticoid-mediated inhibition of inflammatory cytokine production by lipopolysaccharide-stimulated macrophages.[36] This indicates that MIF is a critical component of the immunological and endocrinological systems, and that it acts together with glucocorticoids to regulate immunity and inflammation. Glucocorticoids are known to have profound effects on the proliferation and function of osteoblasts, observed as the reduction of alkaline phosphatase activity, type I collagen and osteocalcin production.[37–39] That is, MIF may act in an autocrine mode on osteoblasts to alter their functions induced by glucocorticoids. Hence, MIF is considered to be a vital component in various osseous pathophysiological conditions. Further investigation of this topic is currently under way.

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